



**Europäisches  
Patentamt**

**European  
Patent Office**

**Office européen  
des brevets**

RECEIVED

18 MAR 2004

WIPO

PCT

**Bescheinigung**

**Certificate**

**Attestation**

Die angehefteten Unterla-  
gen stimmen mit der  
ursprünglich eingereichten  
Fassung der auf dem näch-  
sten Blatt bezeichneten  
europäischen Patentanmel-  
dung überein.

The attached documents  
are exact copies of the  
European patent application  
described on the following  
page, as originally filed.

Les documents fixés à  
cette attestation sont  
conformes à la version  
initialement déposée de  
la demande de brevet  
européen spécifiée à la  
page suivante.

**Patentanmeldung Nr. Patent application No. Demande de brevet n°**

03077237.0

**PRIORITY  
DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**R C van Dijk**



Anmeldung Nr:  
Application no.: 03077237.0  
Demande no:

Anmeldetag:  
Date of filing: 17.07.03  
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Applied Research Systems ARS Holding N.V.  
Pietermaai 15  
Curacao  
ANTILLES NEERLANDAISES

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
Si aucun titre n'est indiqué se referer à la description.)

CC-Chemokine mutants against liver diseases

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)  
revendiquée(s)  
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/  
Classification internationale des brevets:

A61K38/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of  
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL  
PT RO SE SI SK TR LI

- 1 -

**CC-CHEMOKINE MUTANTS AGAINST LIVER DISEASES****FIELD OF THE INVENTION**

5 The present invention relates to novel therapeutic applications of specific CC-chemokine mutants.

**BACKGROUND OF THE INVENTION**

Chemokines are secreted pro-inflammatory proteins of small dimensions (70-130  
10 amino acids) mostly involved in the directional migration and activation of cells, especially the extravasation of leukocytes from the blood to tissue localizations needing the recruitment of these cells (Baggiolini M et al., 1997; Rossi D and Zlotnik A, 2000; Fernandez EJ and Lolis E, 2002). Usually chemokines are produced at the site of an injury, inflammation, or other tissue alteration in a paracrine or autocrine fashion,  
15 triggering cell-type specific migration and activation.

Depending on the number and the position of the conserved cysteines in the sequence, chemokines are classified into C-, CC-, CXC- and CX<sub>3</sub>C-chemokines. Inside each of these families, chemokines can be further grouped according to the homology of the entire sequence, or of specific segments.

20 A series of heptahelical G-protein coupled membrane receptors, are the binding partners that allow chemokines to exert their biological activity on the target cells, which present specific combinations of receptors according to their state and/or type. An unified nomenclature for chemokine ligands and receptors, which were originally named by the scientists discovering them in a very heterogeneous manner, has been  
25 proposed to associate each of these molecule to a systemic name including a

- 2 -

progressive number: CCL1, CCL2, etc. for CC chemokines; CCR1, CCR2, etc. for CC chemokines receptors, and so on.

5 The physiological effects of chemokines result from a complex and integrated system of concurrent interactions. The receptors often have overlapping ligand specificity, so that a single receptor can bind different chemokines, as well a single chemokine can bind different receptors. In particular, N-terminal domain of chemokines is involved in receptor binding and N-terminal processing can either activate chemokines or render chemokines completely inactive.

10 Amongst all the chemokines characterized so far, chemokines, such as CCL5 (also known as RANTES; Appay V and Rowland-Jones SL, 2001) and CCL3 (also known as MIP-1alpha, US 6,355,476) have been intensively studied to identify therapeutically useful molecules. Variants of CC-chemokines, missing up to nine N-terminal amino acids, have been tested for their activity as inhibitors or antagonists of the naturally occurring forms. These molecules are inactive on monocytes and are  
15 useful as receptor antagonists (Gong JH et al., 1996; WO 99/16877). Alternatively, N-terminal extension of the mature CCL-chemokine with one Methionine results in almost complete inactivation of the molecule, which also behaves as an antagonist for the authentic one (WO 96/17935).

20 Moreover, in order to perform structure-function analysis of CC-chemokines, variants containing substitutions or chemical modifications in different internal positions, as well as CC-chemokine derived peptides, have been tested for the interactions with receptors or other molecules. Some of these variants have been disclosed as having considerably altered binding properties, and sometimes they are active as CC-chemokine antagonists, having potential therapeutic applications in the  
25 treatment of HIV infection and some inflammatory or allergic diseases (WO 99/33989;

BEST AVAILABLE COPY

- 3 -

Nardese V et al., 2001). In particular, the binding determinants and the physiological relevance of the interactions of chemokines, by the means of specifically positioned basic residues, with Glycosaminoglycans (GAGs) has been intensively studied (WO 02/28419; Vives R et al., 2002; Martin L et al., 2001; Koopmann W and Krangel MS, 5 1997).

Even though there are potential drawbacks in using chemokines as therapeutic agents (tendency to aggregate, promiscuous binding), these molecules offer the possibility for therapeutic intervention in pathological conditions associated to such processes, in particular by inhibiting / antagonizing specific chemokines and their 10 receptors at the scope to preventing the excessive recruitment and activation of cells, in particular leukocytes, for a variety of indications related to inflammatory and autoimmune diseases, cancers, and bacterial or viral infections (Schneider GP et al., 2001, Baggiolini M, 2001; Godessart N and Kunkel SL, 2001).

The possible therapeutic applications of chemokine-related compounds against 15 hepatic diseases have been intensively studied, as recently reviewed (Ajuebor MN et al., 2002; Marra F, 2002). In particular, liver specific inflammation is mediated by activated CD4(+) T cells and driven by an upregulation of the hepatic expression of IFNgamma, but the mechanisms governing T cell migration from the blood into tissues during T cell-mediated hepatitis remains incompletely understood, since the endogenous mediators 20 that promote the recruitment of T cells to the liver during T cell-mediated liver diseases have been poorly characterized.

It has been demonstrated that some chemokines are highly expressed and capable of recruiting the liver-infiltrating lymphocytes in hepatitis-related animal models (acetaminophen-induced, Concanavalin A-induced, adenovirus-induced, or hepatitis B 25 virus-specific), suggesting a role of these molecules in the development of hepatitis

BEST AVAILABLE COPY

- 4 -

(Lichterfeld M et al., 2002; Katsumitsu A et al., 2002; Bautista AP, 2002; Dambach D et al., 2002; Lalor PF et al., 2002; Kakimi K et al., 2001; Tamaru M et al., 2000; Hogaboam CM et al., 2000; Kusano F et al., 2000; Fisher NC et al., 1999; Miyaguchi S et al., 1997).

5        Some broad spectrum CC-chemokine antagonists were disclosed in connection to hepatic diseases (WO 00/73327; WO 01/58916; US 6,495,515). CXC chemokines are capable to induce rapid hepatocyte proliferation and liver regeneration after injury (WO 01/10899). CCR1 antagonists can be used for inhibiting graft-related or ischemia/reperfusion-related liver dysfunctions (WO 00/44365). However, prior art fails  
10 to describe any therapeutic efficacy of specific CCL3 / CCL5 chemokine mutants having antagonistic properties, for the treatment of T cell mediated, inflammatory, autoimmune, and/or fibrotic liver diseases.

#### SUMMARY OF THE INVENTION

15        It has been surprisingly found that a CCL5/RANTES and CCL3/MIP-1alpha have specific role in T cell mediated liver diseases. In particular CCL5/RANTES mutants having antagonistic properties counteracts effectively liver injury in an animal model. These evidences demonstrate the possibility of using CCL5/RANTES and/or CCL3/MIP-1alpha mutants having antagonistic activity in the treatment of T cell  
20 mediated, inflammatory, autoimmune, and/or fibrotic liver diseases.

Other features and advantages of the invention will be apparent from the following detailed description.

- 5 -

**DESCRIPTION OF THE FIGURES**

Figure 1: time course analysis of the effect of Concanavallin A (Con A) administration on serum alanine transaminase (ALT; A) and on hepatic MIP-1alpha (B) levels in mice.

5 Figure 2: effect of the treatment with Concanavallin A or PBS on MIP-1alpha control (MIP-1α WT) and knock-out (MIP-1α KO) mice on serum ALT (A) or hepatic IFN-gamma (B) levels in mice. The levels were measured 8 hours after the administration of PBS or Con A. The asterisk indicates the statistical significance of the measured difference ( $P < 0.05$ ) when compared to the  
10 PBS-treated controls.

Figure 3: effect of the administration of Met-RANTES on ALT (A) or hepatic IFN-gamma (B) levels in Concanavallin A-treated mice. The levels were measured 8 hours after the administration of PBS or Con A. The asterisk indicates the statistical significance of the measured difference ( $P < 0.05$ )  
15 when compared to the PBS-treated controls.

Figure 4: comparison of the number of IFN-gamma-producing CD4(+) T cells in CCl3/MIP-1alpha control (MIP-1α WT) and knock-out (MIP-1α KO) mice (A) and in control or Met-RANTES-pretreated mice (B) in the Concanavallin A hepatitis model. The levels were measured 8 hours after the  
20 administration of PBS or Con A. The asterisk indicates the statistical significance of the measured difference ( $P < 0.05$ ) when compared to controls.

Figure 5: effect of the administration of triple 40's CCL5/RANTES mutant on ALT level in Concanavallin A-treated mice. The levels were measured 8 hours  
25 after the administration of PBS or Con A. The asterisk indicates the

- 6 -

statistical significance of the measured difference ( $P < 0.05$ ) when compared to the PBS-treated controls.

### DETAILED DESCRIPTION OF THE INVENTION

5 The main object of the present invention is the use of a CCL3/MIP-1alpha and/or of a CCL5/RANTES mutant having antagonistic properties for the treatment of T cell mediated, inflammatory, autoimmune, and/or fibrotic liver diseases.

The present invention is based on the observation that the CCL3/MIP-1alpha and CCL5/RANTES are not only upregulated in a murine T Cell-mediated hepatitis model,  
10 as many other pro-inflammatory molecules, but biochemical indexes associated to liver dysfunction can be considerably improved by administering CCL3 and/or CCL5 mutants having antagonistic properties. These mutants can be used singularly, simultaneously, or in a sequential manner

In particular, preferred CCL5-chemokine mutants which can be used are the ones  
15 disclosed in the prior art under the names of Met-RANTES (SEQ ID NO: 1) and triple 40's RANTES mutant (SEQ ID NO: 2). Mutants of MIP-1alpha having similar properties are known in the art (Koopmann W and Krangel MS, 1997). It is however evident that any other CCL3 and CCL5 mutants having such antagonistic activity, such as the ones having a modified N-terminal sequence or reduced GAG-binding properties resulting  
20 from the substitution of the same residues disclosed in the prior art but with a different amino acid (i.e. the basic residue is substituted with a non-polar amino acid other than Alanine or the acid residue) are intended within the scope of the invention.

Several evidences support an important role for CCL3 and CCL5 in the pathophysiology of several diseases including multiple sclerosis, inflammatory lung  
25 injury and allergic diseases. However, the role played by CCL3 and CCL5 in

BEST AVAILABLE COPY



- 7 -

modulating hepatic inflammation and the mechanisms underlying this modulation in T cell-mediated liver diseases was not understood so far. The above cited prior art on CCL5 and CCL3 mutants does not disclose that such molecules can provide a therapeutic effect against T cell mediated, inflammatory, autoimmune, and/or fibrotic liver diseases. As there are currently therapies only partially effective and/or acceptable for treating diseases such as alcoholic liver diseases, viral or autoimmune hepatitis, CCL3 / CCL5 chemokine mutants represent alternative therapeutic compounds possibly better accepted and efficient than the current therapies

The wording "a reduced GAG-binding activity" or "GAG-binding defective" means that the CCL3 / CCL5 chemokine mutants have a lower ability to bind to GAGs, i. e. a lower percentage of each of these mutants bind to GAGs (like heparin sulphate) with respect to the corresponding wild-type molecule, as measured with the assays in the above cited prior art disclosing such mutants.

In addition to the mutation at the specific positions leading to the decreased affinity for GAGs, the CCL3 / CCL5 chemokine mutants may include other modifications with respect to the wild-type molecule, generating active mutants of said CCL3 / CCL5 chemokine mutants in which one or more amino acids have been added, deleted, or substituted. These additional modifications should be intended to maintain, or even improve, the properties of CCL3 and CCL5 mutants for the uses characterized in the present invention, making them equally useful for treating liver inflammatory and/or fibrotic diseases.

CCL3 and CCL5 mutants can be prepared by chemical synthesis, by site-directed mutagenesis techniques, or any other known technique suitable thereof, which provide a finite set of substantially corresponding mutated or shortened peptides or polypeptides which can be routinely obtained and tested by one of ordinary skill in the

- 8 -

art using the teachings presented in the prior art and in the Examples of the present patent application. Similar compounds may also result from conventional mutagenesis technique of the encoding DNA, from combinatorial technologies at the level of encoding DNA sequence (such as DNA shuffling, phage display/selection), or from  
5 computer-aided design studies based on the tridimensional structure and other functional assays of chemokines, for example with or without the presence of GAGs (Rajaratnam K, 2002; Vives R et al., 2002; Martin L et al., 2001; Koopmann W and Krangel MS, 1997).

In accordance with the present invention, preferred changes in these CCL3 and  
10 CCL5 active mutants are commonly known as "conservative" or "safe" substitutions, that is, with amino acids having sufficiently similar chemical properties, in order to maintain the structure and the biological function of the CCL3 / CCL5 chemokine mutant. It is clear that insertions and deletions of amino acids may also be made in the above defined sequences without altering their function, particularly if the insertions or  
15 deletions only involve a few amino acids, e.g., under ten, and preferably under three, and do not remove or displace amino acids which are critical to the functional conformation of a protein or a peptide.

The literature provide many models on which the selection of conservative amino acids substitutions can be performed on the basis of statistical and physico-chemical  
20 studies on the sequence and/or the structure of natural protein (Rogov SI and Nekrasov AN, 2001). Protein design experiments have shown that the use of specific subsets of amino acids can produce foldable and active proteins, helping in the classification of amino acid "synonymous" substitutions which can be more easily accommodated in protein structure, and which can be used to detect functional and

- 9 -

structural homologs and paralogs (Murphy LR et al., 2000). The synonymous amino acid groups and more preferred synonymous groups are those defined in Table I.

Alternatively, active CCL3 / CCL5 chemokine mutants may contain on or more non-natural, amino acid derivatives being "synonymous" to a natural amino acid, are those defined in Table II. By "amino acid derivative" is intended an amino acid or amino acid-like chemical entity other than one of the 20 genetically encoded naturally occurring amino acids. In particular, the amino acid derivative may contain substituted or non-substituted alkyl linear, branched, or cyclic moieties, and may include one or more heteroatoms. The amino acid derivatives can be made *de novo* or obtained from commercial sources (Calbiochem-Novabiochem AG; Bachem). Various methodologies for incorporating unnatural amino acids derivatives into proteins, using both *in vitro* and *in vivo* translation systems, to probe and/or improve protein structure and function are disclosed in the literature (Dougherty DA, 2000).

The term "active" means that such alternative compounds should maintain the therapeutic properties of the CCL3 / CCL5 chemokines mutants against liver inflammatory and/or fibrotic diseases as described in the present invention, and should be as well pharmaceutically acceptable and useful.

In another embodiment, a polypeptide comprising the CCL3 /CCL5 chemokine mutant and an amino acid sequence belonging to a protein sequence other than the corresponding CCL3 / CCL5 chemokine can be also used for treating liver inflammatory and/or fibrotic diseases. The heterologous sequence is intended to provide additional properties without considerably impairing the therapeutic activity. Examples of such additional properties are an easier purification procedure, a longer lasting half-life in body fluids, an additional binding moiety, the maturation by means of an endoproteolytic digestion, or extracellular localization. This latter feature is of

- 10 -

particular importance for defining a specific group of fusion or chimeric proteins included in the above definition since it allows the CCL3 / CCL5 chemokine mutants to be localized in the space where not only where the isolation and purification of these polypeptides is facilitated, but also where CCL3 / CCL5 chemokines naturally interact with receptors and other molecules. Design of the moieties, ligands, and linkers, as well methods and strategies for the construction, purification, detection and use of fusion proteins are widely discussed in the literature (Nilsson J et al., 1997; "Applications of chimeric genes and hybrid proteins" Methods Enzymol. Vol. 326-328, Academic Press, 2000; WO 01/77137).

Additional protein sequences which can be used to generate the CCL3/CCL5 antagonists can be chosen amongst extracellular domains of membrane-bound protein, immunoglobulin constant regions, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins. The choice of one or more of these sequences to be fused to the CCL3 / CCL5 chemokine mutants is functional to the desired liver-related use, delivery and/or preparation method.

CCL3 / CCL5 chemokine mutants can be also provided for the treatment of liver inflammatory and/or fibrotic diseases in the form of the corresponding active precursor, salts, derivatives, conjugates or complexes. These alternative forms may be preferred according to the desired method of delivery and/or production.

The "precursors" are compounds which can be converted into the compounds of present invention by metabolic and enzymatic processing prior or after the administration to the cells or to the organism.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and

BEST AVAILABLE COPY

- 11 -

include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the peptides and polypeptides of the invention or their analogs.

The term "fractions" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N- or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups. Alternatively, the derivatives may contain sugars or phosphates groups linked to the functional groups present on the lateral chains of the amino acid moieties. Such molecules can result from *in vivo* or *in vitro* processes which do not normally alter primary sequence, for example chemical derivatization of peptides (acetylation or carboxylation), phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes).

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N- or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups

- 12 -

and are formed with acyl-groups as for example alkanoyl- or aroyl-groups. Alternatively, useful conjugates or complexes of the CCL3 / CCL5 chemokine mutants can be generated by using molecules and methods known in the art for improving the detection of the interaction with other proteins (radioactive or fluorescent labels, biotin),  
5 therapeutic efficacy (cytotoxic agents, isotopes), or drug delivery efficacy, such as polyethylene glycol and other natural or synthetic polymers (Pillai O and Panchagnula R, 2001). In the latter case, a site-directed modification of an appropriate residue, present in the natural sequence or introduced by mutating the natural sequence, at an internal or terminal position, can be introduced. Similar modifications have been  
10 already disclosed for chemokines (WO 02/04499; WO 02/04015; Vita C et al., 2002).

Any residue can be used for attachment, provided it has a side-chain amenable for polymer attachment (i.e., the side chain of an amino acid bearing a functional group, e.g., lysine, aspartic acid, glutamic acid, cysteine, histidine, etc.). Alternatively, a residue at these sites can be replaced with a different amino acid having a side chain  
15 amenable for polymer attachment. Polymers suitable for these purposes are biocompatible, namely, they are non-toxic to biological systems, and many such polymers are known. Such polymers may be hydrophobic or hydrophilic in nature, biodegradable, non-biodegradable, or a combination thereof. These polymers include natural polymers (such as collagen, gelatin, cellulose, hyaluronic acid), as well as  
20 synthetic polymers (such as polyesters, polyorthoesters, polyanhydrides). Examples of hydrophobic non-degradable polymers include polydimethyl siloxanes, polyurethanes, polytetrafluoroethylenes, polyethylenes, polyvinyl chlorides, and polymethyl methacrylates. Examples of hydrophilic non-degradable polymers include poly(2-hydroxyethyl methacrylate), polyvinyl alcohol, poly(N-vinyl pyrrolidone), polyalkylenes,

BEST AVAILABLE COPY

- 13 -

polyacrylamide, and copolymers thereof. Preferred polymers comprise as a sequential repeat unit ethylene oxide, such as polyethylene glycol (PEG).

The preferred method of attachment employs a combination of peptide synthesis and chemical ligation. Advantageously, the attachment of a water-soluble polymer will  
5 be through a biodegradable linker, especially at the N-terminal region of a protein. Such modification acts to provide the protein in a "pro-drug" form, that, upon degradation of the linker releases the protein without polymer modification.

The CCL3 / CCL5 chemokine mutants may be prepared by any appropriate procedure in the art, such as recombinant DNA-related technologies involving the  
10 expression in Eukaryotic cells (e.g. yeasts, insect or mammalian cells) or Prokaryotic cells. Detailed methods for producing the GAG-binding defective CCL3 / CCL5 chemokine mutants can be found in the prior art originally disclosing them (WO 02/28419), as well as in other literature featuring protocols for chemokine production (Edgerton MD et al., 2000) or common molecular biology techniques for the production  
15 of recombinant proteins in Prokaryotic or Eukaryotic host cells, such as some titles in the series "A Practical Approach" published by Oxford University Press ("DNA Cloning 2: Expression Systems", 1995; "DNA Cloning 4: Mammalian Systems", 1996; "Protein Expression", 1999; "Protein Purification Techniques", 2001).

Alternatively the CCL3 / CCL5 chemokine mutants may be prepared by any  
20 other well known procedure in the art, in particular, by the well established chemical synthesis procedures, which can be efficiently applied on these molecule given the short length. Totally synthetic chemokines, also containing additional chemical groups, are disclosed in the literature (Brown A et al., 1996; Vita C et al., 2002).

Examples of chemical synthesis technologies are solid phase synthesis and  
25 liquid phase synthesis. As a solid phase synthesis, for example, the amino acid

- 14 -

corresponding to the N-terminus of the peptide to be synthesized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the C-terminus to the N-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl<sub>2</sub>-Bzl (2,6-dichlorobenzyl) for the amino groups; NO<sub>2</sub> (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino groups; and tBu (t-butyl) for the hydroxyl groups). After the synthesis, the desired peptide is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or trifluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method. Finally, the intact full-length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding CCL3 / CCL5 chemokine mutants.

Purification of the natural, synthetic or recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the

BEST AVAILABLE COPY



- 15 -

Invention is affinity chromatography using monoclonal antibodies, heparin, or any other suitable ligand that can bind the target protein at high efficiency and can be immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by means of this ligand while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength. Alternatively, HPLC (High Performance Liquid Chromatography) can be also used.

Another object of the present invention is the use of a CCL3 and/or CCL5 chemokine mutant having antagonistic properties in the preparation of a pharmaceutical composition for the treatment of T cell mediated, inflammatory, autoimmune, and/or fibrotic liver diseases, in particular when formulated in combination with pharmaceutically acceptable carriers, excipients, stabilizers, adjuvants, or diluents. These compositions may contain only one CCL3/CCL5 mutant or a combination of them.

A non-limitative list of disorders involving hepatic damage in which the CCL3 / CCL5 chemokine mutant having antagonistic activities can be used includes alcoholic liver diseases (cirrhosis, steatosis), a viral hepatitis, an autoimmune hepatitis, or any other fibrotic, T Cell mediated liver degeneration.

Still another object of the present invention are methods for the treatment or prevention of liver inflammatory and/or fibrotic diseases, comprising the administration of an effective amount of a CCL3 and/or a CCL5 chemokine mutant having antagonistic properties.

The CCL3 / CCL5 mutants may be used alone, or with another therapeutic composition acting synergically or in a coordinated manner with them in the treatment of liver inflammatory and/or fibrotic diseases. For example, similar synergistic

- 16 -

properties of CCL5 chemokine mutants have been demonstrated in combination with cyclosporin (WO 00/16796).

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of the liver pathology. The effective amount will depend on the route of administration and the condition of the patient.

The pharmaceutical compositions may be formulated in any acceptable way to meet the needs of the mode of administration for treating liver diseases. For example, the use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration, are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001).

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. Carriers can be selected also from starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the various oils, including those of petroleum, animal, vegetable or synthetic origin (peanut oil, soybean oil, mineral oil, sesame oil). For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

Besides the pharmaceutically acceptable carrier, the compositions of the invention can also comprise minor amounts of additives, such as stabilizers, excipients, buffers and preservatives that may facilitate the processing of the active compounds into preparations which can be used pharmaceutically. Moreover, these compositions

BEST AVAILABLE COPY

- 17 -

may contain another active ingredient which can act synergically or in a coordinated manner with the CCL5 / CCL3 mutants.

In view of the claimed uses, any drug delivery method allowing the targeting of the CCL5 / CCL3 mutant to the liver is preferred. Similar methods are known in the prior and may involve the conjugation of the CCL3 / CCL5 chemokine mutant with galactosylated or mannosylated albumin (Chuang VT et al., 2002) or the synthesis of polymeric nanoparticles from a sugar-containing conjugate composed of lactobionic acid, diamine-terminated polyethylene glycol) and cholic acid (Kim IS and Kim SH, 2002).

The administration of such active ingredient may be by intravenous, intramuscular or subcutaneous route. Other routes of administration, which may establish the desired effects of the respective ingredients in the liver, are comprised by the present invention. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, oral, or buccal routes. The pharmaceutical compositions of the present invention can also be administered in sustained or controlled release dosage forms, including depot injections, osmotic pumps, and the like, for the prolonged administration of the polypeptide at a predetermined rate, preferably in unit dosage forms suitable for single administration of precise dosages.

Parenteral administration can be by bolus injection or by gradual perfusion over time. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for

- 18 -

example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran.

5 Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99.99 percent, preferably from about 20 to 75 percent of active compound together with the excipient.

10 The optimal dose of active ingredient may be appropriately selected according to the route of administration, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled.

15 Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage, which is the same, less than, or greater than the initial or previous dose administered to the individual.

20 The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions that can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

BEST AVAILABLE COPY

- 19 -

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

### EXAMPLES

**Example 1: relevance of MIP-1alpha expression in the Concanavallin A mouse model**

#### Materials & Methods

##### *Mice*

Pathogen-free male C57BL/6J mice and CCL3/MIP-1alpha knock-out C57BL/6J mice were purchased (Jackson Laboratories; Charles River Breeding Farms).

##### *Con A-induced hepatitis model.*

Mice (body weight of 21-23 grams; 5-6 weeks old) were injected intravenously with freshly prepared Concanavallin A (Con A; 13.5 mg/kg; Sigma) in 0.1 ml Phosphate Buffer Saline (PBS), or with 0.1 PBS only (vehicle control).

The levels of serum alanine transaminase (ALT) were measured using a commercial kit (Sigma). Blood was collected from mice at 30 minutes, 90 minutes, 8 hours and 24 hours after Con A administration and under halothane anesthesia.

In a separate set of experiments, the levels of hepatic CCL3/MIP-1alpha or IFNgamma were determined in control and CCL3/MIP-1alpha knock-out mice that were injected with Con A or PBS only. Mice were sacrificed at the indicated time points after Con A or PBS injection and livers were perfused with ice-cold sterile PBS to remove blood elements. Individual perfused livers were homogenised in ice-cold PBS buffer containing protease cocktail inhibitor (Sigma) immediately after their removal, tissue homogenates were centrifuged twice, and the supernatants filtered through a 0.45-mm filter and stored at -80°C until used for protein determination. Hepatic CCL3/MIP-1alpha and IFNgamma levels were measured by specific murine ELISA following a

- 20 -

published protocol (Bone-Larson CL et al., 2001), and using commercial antibodies (R&D Systems). Total protein concentration in the homogenates was calculated using a commercial protein colorimetric assay (Bio-Rad Laboratories).

#### *Statistical Analysis*

5 All data are shown as Mean  $\pm$  Standard Deviation. For comparisons of means between 2 experimental groups (n=4-10 mice per group) a Student's unpaired t-test was used, considering P value  $<0.05$  statistically significant. Statistical analyses were performed using GraphPad InStat (version 3.00) Software.

#### Results

10 Concanavalin A (Con A)-induced liver injury depends on the activation of recruitment of CD4(+) T cells by macrophages. In general, T cells are the driving force underlying immunologically mediated hepatic disorders, making therefore the Con-A model highly relevant for studying the pathophysiology of diseases such as autoimmune or acute hepatitis (Takeda K et al., 2000; Tiegs G et al., 1992).

15 The effects of Con A on liver function can be evaluated by measuring the serum concentration of liver-specific enzymes, such alanine aminotransferase (ALT), which is contained in hepatocytes and released into serum when these cells are damaged. ALT is the most widely used marker in humans and animals to document damage and destruction of liver cells (as in hepatitis), and, generally, ALT concentration correlates  
20 with histological degenerative changes.

A time course experiments on normal mice was performed to verify if there is any correlation between ALT levels and hepatic CCL3/MIP-1 $\alpha$  protein concentration. The data demonstrated that maximum hepatic injury, as evaluated by using ALT concentration, and peak hepatic CCL3/MIP-1 $\alpha$  levels can be both observed at 8  
25 hours after intravenous administration of a single dose of Con A (13.5 mg/kg). This

BEST AVAILABLE COPY

- 21 -

compound caused a more than 100-fold increase of ALT levels (Figure 1A) paralleled by a more than five-fold increase of hepatic CCL3/MIP-1alpha protein expression (Figure 1B) above control PBS injection.

The effect of Con A administration in transgenic mice lacking the CCL3/MIP-1alpha gene (Cook DN et al., 1995) to verify if CCL3/MIP-1alpha deficiency impairs the development of Con A-induced hepatic injury. In fact, CCL3/MIP-1alpha knock-out mice exhibited significantly less hepatic injury 8 hours after the Con A injection relative to control mice, as demonstrated biochemically by a significant reduction (approx. five-fold) in ALT level (Figure 2A). Moreover, hepatic concentration of IFNgamma, one of the cytokines implicated in the pathogenesis of Con A-induced hepatitis since it is produced by CD4(+) T cells recruited to the liver (Mizuhara H et al., 1996), is significantly lowered in CCL3/MIP-1a knock-out mice at 8 hours after Con A administration, when compared to control mice (Figure 2B).

These results suggest a crucial pro-inflammatory role for CCL3/MIP-1a in the setting of T cell-mediated hepatic injury induced by Con A in mice, in particular through IFNgamma production. CCL3/MIP-1alpha mutants having antagonistic properties may provide a therapeutic effect in T cell mediated, inflammatory, autoimmune, and/or fibrotic diseases.

## 20 Example 2: efficacy of CCL5 mutants in the Concanavalin A mouse model

### Materials & Methods

#### *Administration of CCL5 mutants*

Triple 40's RANTES mutant and methionylated-RANTES (Met-RANTES) were expressed in *E. coli* and purified as previously described (WO 02/28419; WO 25 96/17935).

- 22 -

Mice were injected intravenously with Met-RANTES or Triple 40's RANTES (30 micrograms/mouse in 0.1 ml PBS) or PBS only (0.1 ml). One hour later, CCL6 mutant or PBS only treated mice were given Con A or PBS, as described in Example 1.

All mice were sacrificed 8 hours after Con A or PBS administration. Assessment of hepatic injury and hepatic IFNgamma expression in each group of mice (n= 3-10) were measured and compared for statistical significance as described in Example 1.

*Detection of IFNgamma-producing CD4(+) T cells*

Perfused livers were obtained from mice and minced in digestive media containing 0.05% collagenase and 0.002% DNase I. After gentle agitation at 37 °C for 30 min, the digest was passed through a nylon mesh and then washed twice with PBS. Cells were then subjected to density gradient centrifugation on Lympholyte-MØ (Cedarlane Lab.) to isolate lymphocytes. Hepatic lymphocytes were resuspended in PBS and viability determined by trypan-blue exclusion.

Flow cytometric immunofluorescence analysis of liver-infiltrating lymphocytes were performed using the isolated hepatic lymphocytes ( $1 \times 10^6$  cells), which were first incubated with a FITC-conjugated anti-mouse CD4 monoclonal antibodies or isotype control (Serotec Inc.) then fixed and permeabilized with cytofix-cytoperm plus (BD Pharmingen). Following permeabilization, cells were incubated with a PE-labelled anti-mouse IFNgamma monoclonal antibodies (clone XMGI.2; BD Pharmingen) for 45 minutes at 4°C and then analyzed by FACS.

For FACS analysis, the lymphocyte population was gated using forward and side scatter characteristics and analyzed using CellQuest software (Becton Dickinson). Due to the lower number of isolated liver-infiltrating lymphocytes obtained from CCL3/MIP-1alpha gene deficient mice after Con A treatment, livers from two of these mice were pooled together to obtain a sufficient number ( $1 \times 10^6$  cells/tube) of lymphocytes for

BEST AVAILABLE COPY



- 23 -

flow cytometry studies. In control, Con A-treated mice, the number of lymphocytes obtained from one mouse liver ( $1 \times 10^6$  cells/tube) was sufficient for FACS staining. Cell viability as determined by trypan dye was >95%.

### Results

5 Both CCL3/MIP-1alpha and CCL5/RANTES bind CCR1 and CCR5, therefore CCL5 mutants having antagonistic effect may provide similar effects on T cell mediated, inflammatory, autoimmune, and/or fibrotic diseases.

Met-RANTES (Proudfoot A et al., 1999; WO 96/17935) and triple 40's RANTES mutant (WO 02/28419) are functional CCL5 antagonists. Mice pre-treated only with  
10 Met-RANTES (i.e., no Con A treatment) did not develop any evidence of liver injury or inflammation at 8 hours compared to PBS-treated mice as demonstrated biochemically (Figure 3A and 3B, left columns). However, pre-treatment with Met-RANTES ameliorates Con A-induced hepatic injury, as observed biochemically by ALT levels which are reduced to statistically relevant lower values and which are comparable to  
15 the one measured in the CCL3/MIP-1alpha defective, Con A-treated mice (Figure 3A and 2A). The same observation was made for hepatic IFNgamma levels (Figure 3B and 2B).

IFNgamma-producing CD4(+) T cells were identified by FACS analysis in Con A-treated mice for both relevant models (MIP-1alpha defective mice and Met-RANTES  
20 pretreated mice) to verify if there the decrease in hepatic IFNgamma levels (see Figure 2A and 2B) is accompanied by a parallel decrease in the recruitment of these cells. The data showed that the specific decrease of CCL3 and CCL5 activity, due either to a genetic alteration or the addition of a specific antagonistic mutant, considerably decrease the recruitment of IFNgamma-producing CD4(+) T cells (Figure 4A and 4B).

- 24 -

Finally, liver injury, as measured biochemically by ALT levels, was also reduced by pre-treating the mice with another CCL5/RANTES mutant having antagonistic properties, the triple 40's RANTES mutant (Figure 5).

In summary, CCL5/RANTES and CCL3/MIP-1alpha play a crucial pro-inflammatory role in Con A-induced hepatitis, a model of T cell-mediated hepatitis, which is mediated through the hepatic recruitment of IFN-gamma producing CD4(+) T cells. Therefore, a specific down-regulation of hepatic CCL5/RANTES and CCL3/MIP-1alpha activated signaling pathways by means of CCL5 and/or CCL3 mutants having antagonistic properties such as the ones known in the literature (WO 02/28419; Proudfoot A et al., 1999; WO 96/17935, Koopmann W and Krangel MS, 1997), justifies a novel therapeutic use of these molecules for the treatment of T cell mediated, autoimmune, inflammatory, and/or fibrotic liver diseases.

The comparison with results obtained using other animal models for such diseases (e.g. acetaminophen-induced hepatitis), may provide further confirmation of the therapeutic applicability of the specific CCL3 and CCL5 antagonistic mutants.

- 25 -

TABLE I

Amino Acid	Synonymous Group	More Preferred Synonymous Groups
Ser	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Arg	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Leu	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Pro	Gly, Ala, Ser, Thr, Pro	Pro
Thr	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Ala	Gly, Thr, Pro, Ala, Ser	Gly, Ala
Val	Met, Phe, Ile, Leu, Val	Met, Ile, Val, Leu
Gly	Ala, Thr, Pro, Ser, Gly	Gly, Ala
Ile	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Phe	Trp, Phe, Tyr	Tyr, Phe
Tyr	Trp, Phe, Tyr	Phe, Tyr
Cys	Ser, Thr, Cys	Cys
His	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Gln	Glu, Asn, Asp, Gln	Asn, Gln
Asn	Glu, Asn, Asp, Gln	Asn, Gln
Lys	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Asp	Glu, Asn, Asp, Gln	Asp, Glu
Glu	Glu, Asn, Asp, Gln	Asp, Glu
Met	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Trp	Trp, Phe, Tyr	Trp

- 26 -

TABLE II

Amino Acid	Synonymous Group
Ser	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Arg	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Leu	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Pro	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Thr	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Ala	D-Ala, Gly, Alb, B-Ala, Acp, L-Cys, D-Cys
Val	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG
Gly	Ala, D-Ala, Pro, D-Pro, Alb, beta.-Ala, Acp
Ile	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Phe	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
Tyr	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Cys	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Gln	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Asn	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Lys	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asp	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Glu	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Met	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val

BEST AVAILABLE COPY

- 27 -

## REFERENCES

- Ajuebor MN et al., *Biochem Pharmacol* 2002, 63:1191-6.
- Appay V and Rowland-Jones SL, *Trends Immunol* 2001, 22: 83-7.
- Baggiolini M et al., *Annu Rev Immunol* 1997, 15: 675-705.
- 5 Baggiolini M, *J Intern Med* 2001, 250: 91-104.
- Bautista AP, *Front Biosci* 2002, 7: a117-25.
- Bone-Larson CL et al., *J Immunol* 2001, 167: 7077-83.
- Brown A et al., *J Pept Sci* 1998, 2: 40-6.
- Chuang VT et al., *Pharm Res* 2002, 19: 569-577.
- 10 Cleland JL et al., *Curr Opin Biotechnol* 2001, 12: 212-9.
- Cook DN et al., *Science* 1995, 269:1583-1585.
- Dougherty DA, *Curr Opin Chem Biol* 2000, 4: 645-652.
- Dambach D et al., *Hepatology* 2002, 35: 1093-103.
- Edgerton MD et al., *Methods Mol Biol* 2000, 138: 33-40.
- 15 Fernandez EJ and Lolis E, *Annu Rev Pharmacol Toxicol* 2002, 42: 469-499.
- Fisher NC et al., *Gut* 1999, 45: 416-20.
- Godessart N and Kunkel SL, *Curr Opin Immunol* 2001, 13: 670-675.
- Gong JH et al., *J Biol Chem* 1996, 271: 10521-7.
- Hogaboam CM et al., *Am J Pathol* 2000, 156: 1245-52.
- 20 Kakimi K et al., *J Exp Med* 2001, 194: 1755-66.
- Katsumitsu A et al., *Cell Immun* 2002, 219: 48-56.
- Kim IS and Kim SH, *Int J Pharm* 2002, 245: 67-73.
- Koopmann W and Krangel MS, *J Biol Chem* 1997, 272: 10103-9.
- Kusano F et al., *Lab Invest* 2000, 80: 416-22.
- 25 Lalor PF et al., *Immunol Cell Biol* 2002, 80: 52-64.

- 28 -

- Lichterfeld M et al., J Infect Dis 2002, 185: 1803-1807.
- Luo B and Prestwich GD, Exp Opin Ther Patents 2001, 11: 1395-1410.
- Marra F, Front Biosci 2002, 7: d1899-914.
- Martin L et al., Biochemistry 2001, 40: 6303-6318.
- 5 Miyaguchi S et al., Hepatology Res 1997, 8: 156-163.
- Mizuhara H et al., Hepatology 1996, 23: 1608-1615.
- Murphy LR et al., Protein Eng 2000, 13:149-52.
- Nilsson J et al., Protein Expr Purif 1997, 11: 1-16.
- Pillai O and Panchagnula R, Cur Opin Chem Biol 2001, 5: 447-451.
- 10 Proudfoot A et al., J Biol Chem 1999, 274: 32478-32485.
- Rajaratnam K, Curr Pharm Des 2002, 8: 2159-2169.
- Rogov SI and Nekrasov AN, Protein Eng 2001, 14: 459-463.
- Rossi D and Zlotnik A, Annu Rev Immunol 2000, 18:217-42.
- Schneider GP et al., Int J Mol Med 2001, 8: 235-44.
- 15 Takeda K et al., Proc Natl Acad Sci U S A 2000, 97: 5498-503.
- Tamaru M et al., Cytokine 2000, 12: 299-308.
- Tiegs G et al., J Clin Invest 1992, 90: 196-203.
- Vita C et al., J Immunol Methods 2002, 266: 53-65.
- Vives R et al., Biochemistry 2002, 41: 14779-89.

- 29 -

### CLAIMS

1. Use of a CCL3 and/or a CCL5 chemokine mutant having antagonistic properties for the treatment of T cell mediated, inflammatory, autoimmune, and/or fibrotic liver diseases.
2. The use of claim 1 wherein the CCL5 mutant has the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.
3. The use of claim 2 wherein said mutant is an active mutant in which one or more amino acids have been added, deleted, or substituted.
4. The use of any of the claims from 1 to 3 wherein said mutant is comprised in a polypeptide additionally comprising an amino acid sequence belonging to a protein sequence other than the corresponding CC-chemokine CCL3 or CCL5.
5. The use of any of the claims from 1 to 4 wherein the CC-chemokine mutant is in the form of an active precursor, salts, derivatives, conjugates or complexes.
6. Use of a CCL3 and/or a CCL5 chemokine mutant having antagonistic properties in the preparation of a pharmaceutical composition for the treatment of T cell mediated, inflammatory, autoimmune, and/or fibrotic liver diseases.
7. The use of any of the preceding claims wherein the liver disease is an alcoholic liver disease, a viral hepatitis, or an autoimmune hepatitis

- 30 -

8. Methods for the treatment or prevention of liver inflammatory and/or fibrotic diseases, comprising the administration of an effective amount of a CCL3 and/or a CCL5 chemokine mutant having antagonistic properties.

BEST AVAILABLE COPY



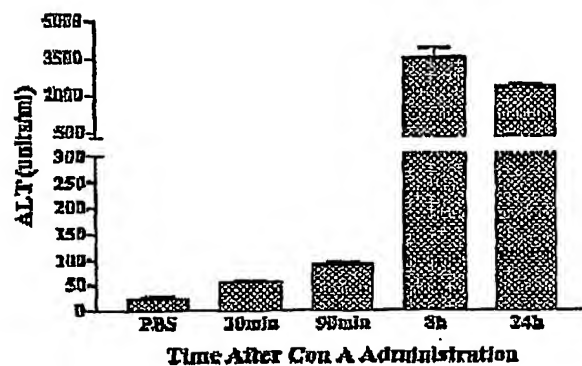
**ABSTRACT**

CC-chemokine mutants having antagonistic properties are effective against T cell mediated, inflammatory, autoimmune, and/or fibrotic liver diseases. Particularly preferred are the mutants of CCL5/RANTES.

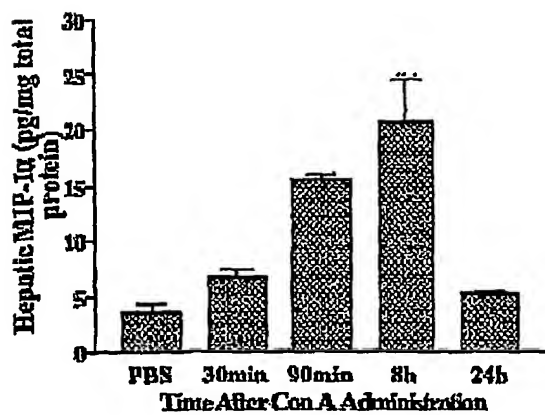
1/5

Figure 1

A)



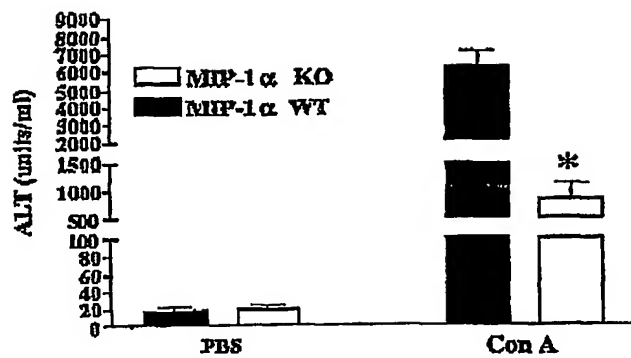
B)



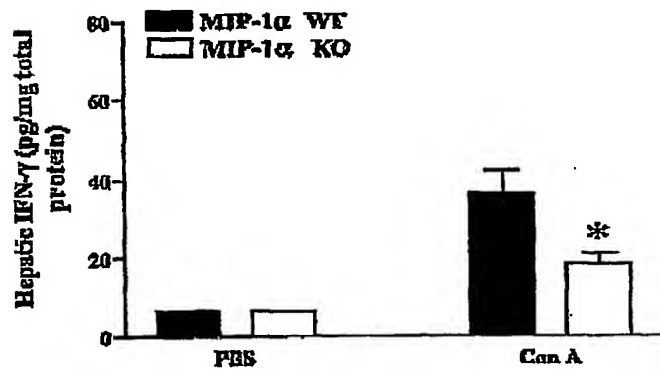
2/5

Figure 2

A)



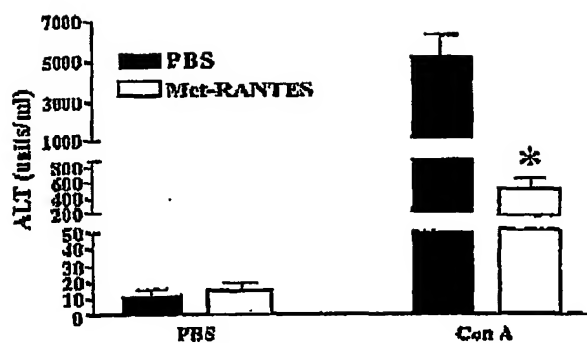
B)



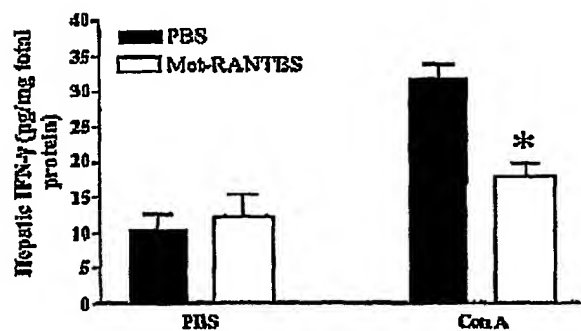
3/5

Figure 3

A)



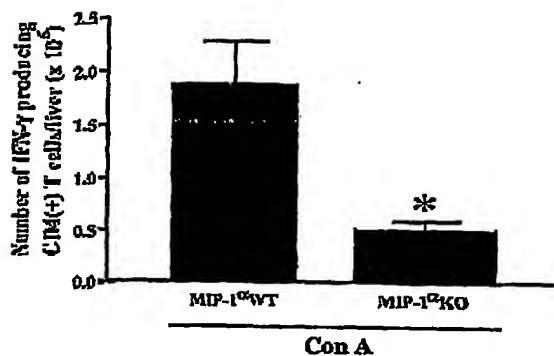
B)



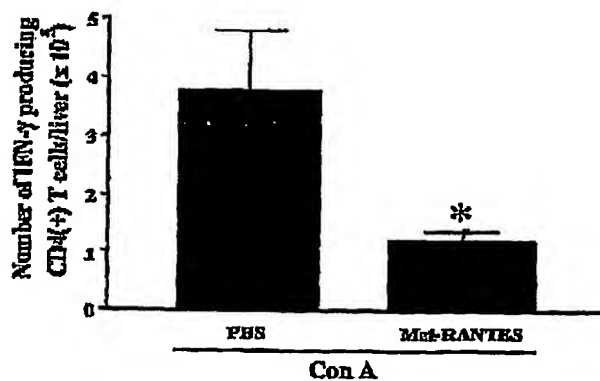
4/5

Figure 4

A)

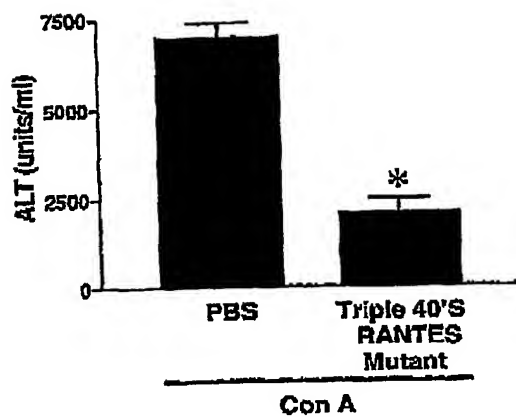


B)



5/5

Figure 5



BEST AVAILABLE COPY

801y.ST25.txt  
SEQUENCE LISTING

&lt;110&gt; Applied Research systems ARS Holding N.V.

&lt;120&gt; CC-CHEMOKINE MUTANTS AGAINST LIVER DISEASES

&lt;130&gt; EP801Y

&lt;160&gt; 2

&lt;170&gt; PatentIn version 3.1

&lt;210&gt; 1

&lt;211&gt; 68

&lt;212&gt; PRT

&lt;213&gt; synthetic construct

&lt;220&gt; Met-RANTES

&lt;221&gt; source

&lt;223&gt;

&lt;400&gt; 1

Met Gly Tyr Ser Ser Asp Thr Thr Pro Cys Cys Phe Ala Tyr Ile Ala  
1 5 10 15Arg Pro Met Pro Arg Ala His Ile Lys Glu Tyr Phe Tyr Thr Ser Gly  
20 25 30Lys Cys Ser Asn Pro Ala Val Val Phe Val Thr Arg Lys Asn Arg Gln  
35 40 45Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg Glu Tyr Ile Asn Ser  
50 55 60Leu Glu Met Ser  
65

&lt;210&gt; 2

&lt;211&gt; 68

&lt;212&gt; PRT

&lt;213&gt; synthetic construct

&lt;220&gt; Triple 40's RANTES

801y.ST25.txt

<221> source  
<223>

<400> 2

Ser Pro Tyr Ser Ser Asp Thr Thr Pro Cys Cys Phe Ala Tyr Ile Ala  
1 5 10 15

Arg Pro Leu Pro Arg Ala His Ile Lys Glu Tyr Phe Tyr Thr Ser Gly  
20 25 30

Lys Cys Ser Asn Pro Ala Val Val Phe Val Thr Ala Ala Asn Ala Gln  
35 40 45

Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg Glu Tyr Ile Asn Ser  
50 55 60

Leu Glu Met Ser  
65



PCT/EP2003/051090

